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CSF1 over-expression has pleiotropic effects on microglia *in vivo*

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Abstract

Macrophage colony stimulating factor (CSF1) is a cytokine that is upregulated in several diseases of the central nervous system (CNS). To examine the effects of CSF1 over-expression on microglia, transgenic mice that over-express CSF1 in the *glial fibrillary acidic protein (GFAP)* compartment were generated. CSF1 over-expressing mice have increased microglial proliferation and increased microglial numbers compared to controls. Treatment with PLX3397, a small molecule inhibitor of the CSF1 receptor CSF1R and related kinases, decreases microglial numbers by promoting microglial apoptosis in both CSF1 over-expressing and control mice. Microglia in CSF1 over-expressing mice exhibit gene expression profiles indicating that they are not basally M1 or M2 polarized, but they do have defects in inducing expression of certain genes in response to the inflammatory stimulus lipopolysaccharide (LPS). These results indicate that the CSF1 over-expression observed in CNS pathologies likely has pleiotropic influences on microglia. Furthermore, small molecule inhibition of CSF1R has the potential to reverse CSF1-driven microglial accumulation that is frequently observed in CNS pathologies, but can also promote apoptosis of normal microglia.

Keywords

Csf1 (Mcsf); microglia; PLX3397; M1 M2 polarization

Introduction

Microglia are CNS resident macrophages that originate from yolk sac derived precursors that take up residence in the CNS during early embryonic development (Ginhoux et al. 2010). Microglial proliferation is observed during neonatal development yet is rare in the normal adult mouse after P20 (Harry and Kraft 2012). However, microglial proliferation can be induced by disease or injury.

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CSF1 is a cytokine for cells of the mononuclear phagocyte system. Depending on the context and the exact cell type studied, CSF1 actions at its receptor CSF1R can promote migration, proliferation, differentiation, survival or polarization of macrophage-lineage cells (Hume and MacDonald 2012). *Csf1r* deficient mice have severe reductions in microglia due to a disruption in microglial development (Erblich et al. 2011; Ginhoux et al. 2010). Some studies of *Csf1* deficient mice have found reduced microglial numbers (Kondo and Duncan 2009; Kondo et al. 2007; Sasaki et al. 2000; Wegiel et al. 1998). IL-34 is a second ligand for CSF1R and *Il-34* deficient mice also have reduced microglial numbers with the reduction in *Il-34* null mice being potentially more severe than in *Csf1* deficient mice (Greter et al. 2012; Kondo and Duncan 2009; Wang et al. 2012). However, these studies did not address if the CSF1R signaling axis is absolutely required for microglial homeostasis in the unperturbed adult CNS.

In addition to regulating their numbers, CSF1 signaling can also influence the phenotype of mononuclear phagocytes. Activated macrophages can be broadly classified as being polarized to an M1 (pro-inflammatory) or M2 (immunosuppressive) phenotype (Sica and Mantovani 2012). *In vitro* studies of human monocyte to macrophage differentiation have suggested that exposure to CSF1 promotes polarization toward an M2-like phenotype (Martinez et al. 2006; Verreck et al. 2004). It has also been shown that mouse macrophages derived from bone marrow cultured in CSF1 are M2-like (Fleetwood et al. 2007). However, the impact of high levels of CSF1 on microglial phenotypes has not been studied *in vivo*.

Increased levels of CSF1, increased microglia and microglial activation are found in many different CNS pathologies including tumors, neurodegenerative diseases and injury (Charles et al. 2012; El Khoury and Luster 2008; Imai and Kohsaka 2002; Loane and Byrnes 2010). To test the hypothesis that increased CSF1 levels impact both microglial numbers and phenotypes *in vivo*, we generated transgenic mice that express the cDNA encoding the secreted isoform of CSF1 under regulation of the tetracycline responsive element (TRE). Crossing TRE-CSF1 to mice that express the tetracycline transactivator (tTA) under control of the *GFAP* promoter (Wang et al. 2004) resulted in increased levels of *Csf1* mRNA and protein compared to controls, an increase in microglial proliferation and an increase in microglial numbers. Treatment with a small molecule inhibitor of CSF1R and related kinases reversed the increase in microglial numbers in CSF1 over-expressing mice and reduced microglial numbers in control mice as well by promoting microglial apoptosis. However, unlike what has been observed *in vitro* for exposure to CSF1 for other macrophage populations, microglia exposed to high levels of CSF1 *in vivo* did not harbor gene expression profiles consistent with M2 polarization, but did have defects in LPS-induced gene expression. In summary, CSF1 over-expression has multiple impacts on microglia *in vivo* and transgenic CSF1 over-expressing mice provide a genetic model system for future studies of the role of increased CSF1 expression and therapeutic CSF1R inhibition in CNS diseases.

MATERIALS/METHODS

Mice

Mouse experiments were performed according to the institutional guidelines for animal care under the approval of the IACUC of the University of Wisconsin, Madison. To generate TRE-CSF1-IRES-GFP the cDNA encoding the secreted isoform (also known as full-length) of murine *Csf1* was obtained from Open Biosystems and PCR amplified to add *Bst*RI and *Bgl*RI restriction sites for cloning. *Csf1* was cloned into TRE-Fgf6-IRES-GFP (eGFP) (White et al. 2006) (generously provided by D. Ornitz) after excision of *Fgf9* with *Sac*RI and *Bam*HI. The TRE-CSF1-IRES-GFP construct was verified by sequencing and a *Not*I digest used to excise the relevant sequence for generating transgenic mice. Pronuclear injections were performed by the University of Wisconsin Carbone Comprehensive Cancer Center (UWCCC) transgenic and mutant animal facility on the C57Bl/6J genetic background. Founders were screened by both PCR (Table 1) and Southern Analysis to detect transgene sequences. Several lines of TRE-CSF1 mice were generated, and the line that expressed the most transgene mRNA as analyzed by qRT-PCR following crossing to GFAP-tTA was used for the experiments reported here. GFAP-tTA mice on the C57Bl/6J genetic background (Wang et al. 2004) were obtained from Jackson laboratories. Throughout the text, CSF1 over-expressing mice refer to the genotype TRE-CSF1; GFAP-tTA. Unless otherwise noted, controls were a combination of littermate GFAP-tTA only, TRE-CSF1 only or mice lacking both transgenes as we have observed no differences in brain *Csf1*, *Iba1* and *Cd11b* levels between these groups (Figure 1B and Supplemental Figure 1A). Furthermore, we do not detect GFP expression in brains or peripheral organs from TRE-CSF1 mice (Supplemental Figure 2). Unless otherwise noted, mice were analyzed at 3–4 weeks of age.

LPS treatment

Mice were intraperitoneally injected with PBS (vehicle) or LPS 5mg/kg (from *E. coli* 011:B4, Sigma) dissolved in PBS. Mice were perfused 3 hours after injection, and processed for immunomagnetic microglial isolation.

Immunomagnetic microglial isolation

Following transcardial perfusion with ice-cold PBS, whole brains (including cerebellum and brain stem) were dissected and weighed. Microglia were isolated as described in detail before (Nikodemova and Watters 2012). Briefly, tissues were dissociated with papain (0.7 mg/ml; Sigma) supplemented with DNase I (50 µg/ml; Worthington) for 25 minutes at 37°C. Myelin was removed by centrifugation in 30% Percoll in PBS at 700g for 15 minutes. After washing in Hank's Balanced Salt Solution, cells were stained with anti-CD11b-PE antibodies (Miltenyi Biotec) in IMAG buffer (PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA) for 10 minutes, followed by a 15 minute incubation with anti-PE microbeads (Miltenyi Biotec). Microglia were separated on MS columns (Miltenyi Biotec) in a magnetic field. The entire procedure, except for the enzymatic dissociation step, was performed at 4°C. The purity of isolated microglia was > 96%. The yield of isolated CD11b⁺ cells was determined by counting an aliquot of the isolate on a hemocytometer utilizing trypan blue exclusion to identify live cells. The viability of cells isolated by this method is more than 93% (Nikodemova and Watters 2012).

RNA isolation and quantitative RT-PCR (qRT-PCR)

For qRT-PCR for M1 and M2 markers, total RNA was extracted from isolated microglia using TRI-Reagent (Sigma). cDNA was synthesized from 0.5 µg of total RNA using MMLV reverse transcriptase (Invitrogen) and a mixture of random primers and oligo(dT) primers (Promega). Quantitative PCR was performed using Power SYBR Green solution (Applied Biosystems) on an ABI 7500 Fast detection system. Gene expression was normalized to 18s rRNA and 2^{-C_t} values calculated. For other studies, RNA was extracted from left brain hemispheres using TRIZOL (Invitrogen) and phenol-chloroform extraction and further purified using the RNeasy Mini Kit (Qiagen). First strand cDNA was generated using an 80:20 mix of polyT:random decamer primers (Ambion Retroscript). Real-time PCR was completed using Step One Plus Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was normalized to *Gapdh* and 2^{-C_t} values calculated. Primer sequences are provided in Table 2. All primers were designed to span introns wherever possible, and primer specificity was assessed through NCBI BLAST analysis prior to use. Single peak dissociation curves with an observed T_m consistent with the expected amplicon length were verified for each sample.

Immunofluorescence

For GFP visualization, mice were transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in 100mM phosphate buffer, pH 7.4. Following overnight fixation in 4% PFA, samples were sucrose sunk, embedded in Optimal Cutting Temperature compound (OCT), frozen and cryosectioned. Otherwise, following transcardial perfusion with PBS, brains were isolated and formalin fixed/paraffin embedded. For all immunofluorescence studies, PBS washes were performed between each step and sections were mounted in Vectashield with DAPI (Vector laboratories) prior to imaging on an Olympus confocal microscope. For CSF1/GFAP double staining, 10 µm sections of formalin fixed/paraffin embedded tissues were de-paraffinized and rehydrated. Antigen retrieval was performed in an antigen unmasking chamber in citrate buffer (Vector laboratories) for 30 seconds at 125°C and 16–19 psi. Sections were blocked in 5% normal rabbit serum (Vector laboratories) at room temperature for one hour and then incubated with goat anti-CSF1 (R&D systems) at 1:25 dilution at 4°C overnight. A biotin labeled anti-goat secondary antibody (Abcam) was used at 1:150 dilution at room temperature for one hour. This was followed by an hour-long incubation with streptavidin-FITC (eBioscience) at 1:200 at room temperature. Tissues were then re-blocked in 5% normal goat serum (NGS) (Vector laboratories) for one hour at room temperature, and then incubated in rabbit anti-GFAP (Abcam) at 1:1000 dilution at 4°C overnight. Anti-rabbit Texas Red secondary antibody (Vector laboratories) was applied to the tissues at 1:125 dilution at room temperature for 30 minutes. For Ki67/IBA1 double labeling, 10 µm sections underwent antigen retrieval as described above. Sections were blocked in M.O.M. kit block (Vector laboratories) at room temperature and incubated with mouse anti-Ki67 (BD Pharmingen, 1:200 dilution) and rabbit anti-IBA1 (Wako, 1:200 dilution) overnight at 4°C in M.O.M. kit diluent. Secondary antibodies were anti-mouse FITC (Abcam, 1:150) and anti-rabbit Texas Red (1:125), and were applied for 30 minutes at room temperature. Ki67⁺ cells were identified and imaged at 60×. A Z series of .8 µm steps was acquired through a 10 µm section and collapsed for quantification using Image J software by an observer blinded to genotype. A cell was

considered IBA1⁺ if the IBA1 signal surrounded and covered the majority of the nucleus. Numbers presented represent the percent of IBA1⁺ cells that are Ki67⁺ (percent of microglia that are proliferating) for a total of four separate 60× fields for each brain region for each mouse. For GFP fluorescence/GFAP immunofluorescence, cryosections were incubated at 37°C for 30 minutes and blocked in 10% NGS at room temperature for 1 hour. GFAP immunofluorescence was performed as described above, with the modification that secondary antibody incubation time was 30 minutes. To increase sensitivity for detecting GFP in experiments shown in Supplementary Figure 2, cryosections were blocked utilizing the M.O.M. kit and incubated at 4°C overnight with mouse anti-GFP at 1:50 (Santa Cruz Biotechnology) and rabbit anti-GFAP at 1:1000. Biotinylated anti-mouse at 1:250 (Vector Laboratories) followed by streptavidin-FITC and anti-rabbit Texas Red secondary antibody (1:125) was applied to the tissues. For TUNEL/IBA1 double labeling, 10 µm sections were subjected to antigen retrieval as described above. TUNEL labeling was performed using the DeadEnd™ Colorimetric TUNEL System (Promega) following the manufacturer's instructions except that proteinase K treatment was omitted and incorporated biotinylated nucleotides were visualized using streptavidin-FITC at 1:200 for 1 hour at room temperature instead of streptavidin-HRP. Slides were subjected to IBA1 immunofluorescence as described above. TUNEL⁺ cells were identified in random 20× fields and were individually examined at higher magnification to determine IBA1 status by an observer blinded to genotype and treatment status. Z series were acquired to verify that TUNEL⁺IBA1⁺ cells do not represent a TUNEL⁺ IBA1⁻ cell being engulfed by a microglia. The total numbers of TUNEL⁺ IBA1⁺ cells from four random 20× fields in the brainstem of each mouse are reported.

IBA1 immunohistochemistry

10 µm sections were de-paraffinized and rehydrated. Antigen retrieval was performed as described above. Tissue sections were quenched in 3% hydrogen peroxide for 10 minutes, washed in PBS and then blocked in 5% NGS overnight at 4°C. Tissues were then incubated in rabbit anti-IBA1 at 1:200 dilution for 3 hours at room temperature. This was followed by a 30 minute incubation with a biotin labeled anti-rabbit secondary antibody (Abcam) at 1:200 dilution at room temperature. The Vectastain ABC reagent (Vector laboratories) was used for antigen signal enhancement and DAB chromogen was used to visualize staining. A Hematoxylin stain was used to visualize nuclei. For IBA1⁺ cell quantification, random 40× fields in the brainstem and midbrain were acquired and quantified using Image J software by an observer blinded to genotype. A cell was considered IBA1⁺ if the IBA1 signal surrounded and covered the majority of the nucleus. Numbers presented represent the total of four separate 40× fields for each brain region for each mouse.

PLX3397 treatment

PLX3397 was generously provided by Plexxikon in chow at a concentration of 290mg/kg of chow (Research Diets, Inc.). Control chow was the same chow without drug. At sacrifice, mice were transcardially perfused with ice-cold PBS. For some mice, left hemispheres were snap frozen for RNA purification. Right hemispheres or whole brains were formalin fixed and paraffin embedded. To examine GFP fluorescence, mice treated for one week were perfused with PBS followed by 4% PFA.

Statistical analysis

Statistical analysis was performed as indicated in each figure legend using Graph Pad Prism software. All error bars represent standard error.

Results

Generation of CSF1 over-expressing mice

To study the impact of elevated levels of CSF1, mice that express CSF1 under the regulation of the tetracycline responsive element (TRE) were generated. The TRE-CSF1 transgene also harbors IRES-GFP sequences (Figure 1A) but for simplicity will hereafter be referred to as TRE-CSF1. In these mice the transgene is expressed only in the presence of the TET-transactivator transcription factor (tTA). Therefore, CSF1 can be expressed in a variety of tissues by crossing to mice expressing tTA under various promoters. To over-express CSF1 in the CNS, TRE-CSF1 mice were crossed to mice that express tTA (“TET OFF” version, activity repressed by tetracyclines) under the control of the *GFAP* promoter (GFAP-tTA) (Wang et al. 2004). qRT-PCR for *Csf1* indicates that GFAP-tTA; TRE-CSF1 doubly transgenic mice (hereafter referred to as “CSF1 over-expressing”) express approximately 4.3 fold more *Csf1* mRNA than controls (Figure 1B). To determine if CSF1 protein is produced in GFAP⁺ cells in double transgenics, dual-immunofluorescence with anti-CSF1 and anti-GFAP antibodies was performed. In this experiment, any GFP fluorescence is quenched due to the formalin fixation/paraffin embedding process, which allowed the use of FITC-conjugated reagents to detect CSF1. CSF1 protein was detected in a subset of GFAP⁺ cells in double transgenic mice but not in controls (Figure 1C). Direct analysis of GFP expression on perfusion-fixed cryo-embedded samples with co-labeling with anti-GFAP antibodies also confirmed transgene expression in a subset of GFAP⁺ cells (Figure 1D). On a subcellular level, GFP fluorescence was sometimes detected in the absence of GFAP immunofluorescence (Figure 1D). This is consistent with previous reports utilizing GFAP-GFP mice and is not unexpected as GFP can diffuse throughout the cell while GFAP is a component of intermediate filaments and therefore has a more restricted subcellular localization (Nolte et al. 2001; Sofroniew and Vinters 2010; Zhuo et al. 1997).

GFAP is expressed outside of the CNS in cell types that include enteric glia, non-myelinating Schwann cells and mesenchymal stellate cells (Sofroniew and Vinters 2010). Consistent with this, some GFP expression was observed outside of the CNS in TRE-CSF1; GFAP-tTA mice but not TRE-CSF1 only mice (Supplemental Figure 2A). However, this expression did not result in a statistically significant difference in *Csf1* levels in intestine, kidney or liver of TRE-CSF1; GFAP-tTA mice compared to controls (Supplemental Figure 2B).

Chi square analysis shows that CSF1 over-expressing mice are born at a ratio that does not deviate from Mendelian inheritance ($p=.1616$), indicating that CSF1 over-expression in the *GFAP* compartment does not adversely impact development. CSF1 over-expressing mice appeared outwardly normal and were fertile. Aging a small number of CSF1 over-expressing mice on a mixed genetic background to one year of age did not reveal any obvious phenotypes.

CSF1 over-expression causes an increase in microglial numbers by promoting microglial proliferation

To investigate if CSF1 over-expression *in vivo* is sufficient to cause an increase in microglial numbers, Cd11b⁺ cells were isolated and counted from six-week old CSF1 over-expressing and control brains. Total numbers of Cd11b⁺ cells were increased approximately 2 fold in CSF1 over-expressing mice (Figure 2A). As brains from CSF1 over-expressing mice weighed slightly less than controls (Figure 2A), this increase was not simply due to an increase in brain mass. qRT-PCR and IHC for additional microglial markers performed in the context of PLX3397 drug studies (see Figure 3 below) confirmed that CSF1 over-expressing mice have increased microglial numbers.

To determine if CSF1 increases microglial numbers by regulating their proliferation, Ki67/IBA1 double immunofluorescence staining was performed (Figure 2B). The percent of IBA1⁺ cells that are Ki67⁺ (percent of microglia proliferating) was quantified in the brainstem and midbrain (Figure 2C), two regions known to exhibit high levels of GFP (transgene) expression in CSF1 over-expressing mice. In control mice, no IBA1⁺Ki67⁺ cells were observed while proliferating microglia were readily observed in CSF1 over-expressing mice. Therefore, CSF1 over-expression in the *GFAP* compartment is sufficient to promote microglial proliferation and an increase in brain microglial numbers.

Pharmacological inhibition of CSF1R with PLX3397 promotes microglial apoptosis and reverses microglial phenotypes in CSF1 over-expressing mice

PLX3397 is a small molecule inhibitor of CSF1R and related kinases (DeNardo et al. 2011) that is able to cross the blood brain barrier (BBB) (Coniglio et al. 2012). PLX3397 is also currently in clinical trials for several cancer types including glioblastoma (clinicaltrials.gov), which are known to express high levels of CSF1 (Bender et al. 2010; Komohara et al. 2008). We therefore sought to determine if PLX3397 is capable of reversing the increase in microglial numbers in CSF1 over-expressing mice by treating three-week old CSF1 over-expressing and control mice with PLX3397 containing chow or control chow for 1 week. qRT-PCR for the microglial markers *Iba1* and *Cd11b* (Figure 3A) on mRNA isolated from whole left brain hemispheres revealed that CSF1 mice treated with control chow harbored approximately 2.5 fold increased levels of these markers compared to control mice, similar to the 2 fold increase in CD11b⁺ cell number observed in these mice at six weeks of age (see Figure 2A). PLX3397 treatment dramatically reduced expression of both *Iba1* and *Cd11b* in CSF1 over-expressing mice and in control mice as well. *Iba1* and *Cd11b* expression levels in PLX3397 treated CSF1 mice actually fell to below normal levels (i.e. control mice treated with control chow). The impact on microglial numbers in CSF1 over-expressing mice was not due to a decrease in expression of the CSF1 transgene as GFP expression and *Csf1* levels were the same in mice treated with control and PLX3397 chow (Supplemental Figure 3). In addition, no changes in the expression of the astrocyte marker *Gfap*, the mature neuron marker *Rbfox3* (NeuN), and the myelinating oligodendrocyte marker *Mog* were observed by qRT-PCR following PLX3397 treatment (Supplemental Figure 4), indicating that PLX3397 is not generally toxic in the CNS.

To further examine the microglial phenotype in response to PLX3397 treatment, IHC for IBA1 was used to quantify microglial numbers in both the brainstem and midbrain (Figure 3B). Quantification of the percentage of cells that are IBA1⁺ verified that in mice receiving control chow, CSF1 over-expressing mice harbor increased numbers of microglia compared to control mice (Figure 3C). PLX3397 treatment decreased IBA1⁺ cells in the midbrain of both CSF1 over-expressing and control mice and in the brainstem of CSF1 over-expressing mice. There was a trend toward a decrease in IBA1⁺ cells in the brainstem of control mice fed PLX3397 chow, but that decrease did not reach statistical significance. In PLX3397 treated CSF1 over-expressing mice, microglial levels were restored to normal levels (i.e. control mice treated with control chow) in the brainstem and were even lower than normal levels in the midbrain.

To determine if microglial apoptosis was responsible for the observed phenotype in PLX3397 treated mice, both CSF1 over-expressing and control mice were treated with PLX3397 for 2 days prior to sacrifice for analysis. IHC for IBA1 revealed that microglia had altered morphology (Figure 3B), indicating that 2 days of drug treatment was sufficient to impact microglial phenotypes. Double labeling with TUNEL and IBA1 was used to detect microglial apoptosis and the number of TUNEL⁺ IBA1⁺ cells (Figure 3D and Supplemental Figure 5) in the brainstem was quantified (Figure 3E). Increased numbers of apoptotic microglia were observed when either control or CSF1 over-expressing mice were treated with PLX3397. Therefore, PLX3397 treatment reduces microglial numbers by promoting their apoptosis in both control and CSF1 over-expressing mice. Interestingly, control mice treated with PLX3397 for two days harbored more apoptotic microglia than did CSF1 over-expressing mice treated with PLX3397, indicating that CSF1 over-expression has some protective effects against the actions of PLX3397. In the absence of PLX3397 treatment there was a trend toward increased microglial apoptosis in CSF1 over-expressing mice compared to controls, however this did not reach statistical significance.

CSF1 over-expression does not promote basal microglial polarization *in vivo* but does cause defects in response to LPS

To determine if exposure to high levels of CSF1 promotes microglial polarization *in vivo*, 6 week-old control and CSF1 over-expressing mice were treated systemically with PBS or LPS. Microglia were then isolated by purification of CD11b⁺ cells and qRT-PCR was utilized to examine expression levels of several markers of M1 and M2 phenotypes (Figure 4). No statistically significant differences were observed in inflammatory markers between PBS treated controls or CSF1 over-expressing mice. However, differences were observed in microglial responses to LPS. Microglia from LPS-challenged CSF1 over-expressing mice expressed less *Il1 β* , *iNos*, *Arg1*, *Il10* and *Ym1* but similar levels of *Il6*, *Tnfa*, and *Tgfb β* compared to LPS-challenged controls. Therefore, CSF1 over-expression does not influence the basal polarization state of microglia, but it does impact their response to LPS.

DISCUSSION

We have generated a genetic model to investigate the impact of increased levels of CSF1 on the CNS *in vivo*. A previous transgenic approach to elucidate the effects of high levels of

CSF1 expression employed the *Csf1r* promoter to drive CSF1 expression (Wei et al. 2006). By IHC, these mice were shown to have increased numbers of cells positive for the macrophage marker F4/80 in several tissues including brain, although the extent of the increase was not explicitly quantified. However, these mice also suffered from lethality prior to sexual maturity so transgenic lines could not be established for further study. Thus, we utilized the TRE promoter and GFAP-tTA transgenic mice to limit expression to GFAP⁺ cells. Although we have exclusively used GFAP-tTA to drive CSF1 expression for this study, the use of other tTA transgenic lines will allow the impact of CSF1 over-expression in other tissues to be elucidated. In support of this, a similar approach was utilized to examine the impact of CSF1 over-expression on breast cancer progression using a tTA line that expresses in salivary and mammary glands (Lin et al. 2001).

Previously, recombinant CSF1 injected directly into the brain (Gomez-Nicola et al. 2013) or delivered systemically (Boissonneault et al. 2009; Gowing et al. 2009; Lalancette-Hebert et al. 2007; Luo et al. 2013) has been used to study the actions of increased CSF1 levels in mouse models of CNS disease or injury. Compared to direct injection into the CNS, a genetic model allows for the effects of CSF1 on the CNS to be assessed without injection-related damage. The CNS actions of systemically delivered CSF1 have been studied in models of disease or injury where there is likely disruption to the BBB (Boissonneault et al. 2009; Gowing et al. 2009; Lalancette-Hebert et al. 2007; Luo et al. 2013), so it is unclear if systemically delivered CSF1 can enter the normal CNS. Therefore, one potential advantage of transgenic CSF1 expression in the *GFAP* compartment is that it allows for expression inside the BBB. In addition, *in vivo* production of CSF1 provides the opportunity for post-translational modifications (Pixley and Stanley 2004) that are likely absent from recombinant preparations derived from bacterial expression systems.

In vitro, most studies have concluded that CSF1 acts as a mitogen for fetal, neonatal and adult microglia (Giulian and Ingeman 1988; Lee et al. 1994; Lee et al. 1993; Liu et al. 2011; Smith et al. 2013; Yamamoto et al. 2010). *In vivo*, CSF1 has been shown to act as a mitogen for putative adult microglia when directly injected in the dorsal hippocampus of a prion disease model (Gomez-Nicola et al. 2013) or when injected systemically into a mouse Alzheimer's disease model (Boissonneault et al. 2009), a model of focal cerebral ischemia (Lalancette-Hebert et al. 2007) and a model of amyotrophic lateral sclerosis (Gowing et al. 2009). Our results are consistent with these observations and indicate that CSF1 *in vivo* can induce microglial proliferation in the absence of additional pathology.

Microglia and/or macrophages invading from the periphery can play both protective and detrimental roles in CNS disease pathology, and these roles likely vary from disease to disease and may also change during disease progression (Bowerman et al. 2013; Gentleman 2013; Giulian and Ingeman 1988). However, because of the putative roles of microglia in promoting neuroinflammation, neurodegeneration and tumor immunosuppression, signaling pathways that influence microglial proliferation, survival and/or activity are considered candidate targets for therapeutic intervention in some CNS diseases and conditions (Bowerman et al. 2013; Ding et al. 2014; Wang et al. 2014). For example, PLX3397, an inhibitor of CSF1R and related kinases is in clinical trials for glioblastoma. Previously, PLX3397 was shown to lower microglial numbers in a mouse glioma allograft tumor model

(Coniglio et al. 2012). Based on *in vitro* data involving co-culture of glioma cells and microglia, this reduction was hypothesized to be due to blockade of microglial recruitment. We found that treatment of mice with PLX3397 caused reductions of microglia by promoting apoptosis. While our paper was under revision, Elmore et al. (2014) also reported that PLX3397 promotes microglial apoptosis in normal adult mice. Our study found that PLX3397 also causes microglial apoptosis in CSF1 over-expressing mice, indicating that the drug may have utility in reducing microglial numbers in CNS diseases where CSF1 is over-expressed. As PLX3397 is currently in clinical trials for glioblastoma, it will be prudent to investigate if PLX3397 can also induce microglial apoptosis in CNS tumors as well.

Studies in a mouse prion disease model found that a CSF1R blocking antibody blocked microglial proliferation but did not cause them to change morphology or undergo apoptosis (Gomez-Nicola et al. 2013). There are several possibilities for the differences observed between this study and treatment with PLX3397. The prion disease state and/or the direct injection into the hippocampus of the CSF1R blocking antibody could have led to the induction of additional factors that promote microglial survival. In addition, a one-time injection of blocking antibody would likely have a more short-lived effect than a week of continued exposure to a small molecule inhibitor.

GW2580, a small molecule inhibitor of CSF1R was also found to inhibit microglial proliferation and to slow disease progression in the same prion disease model (Gomez-Nicola et al. 2013). Recently, the CSF1R small molecule inhibitor BLZ945 was shown to impact the polarization of microglia within gliomas but not their numbers. BLZ945 did reduce microglial numbers in normal brain, however the mechanism was not investigated (Pyonteck et al. 2013). GW2580 and BLZ945 are reported to be highly selective CSF1R inhibitors (Conway et al. 2005; Pyonteck et al. 2013), while PLX3397 inhibits CSF1R (*in vitro* IC₅₀ 20 nM), cKIT (*in vitro* IC₅₀ 10 nM) and to a lesser extent FLT3 (*in vitro* IC₅₀ 160 nM) (DeNardo et al. 2011). Therefore, it is possible that PLX3397 promotes microglial apoptosis through actions on receptor tyrosine kinases other than CSF1R. Previous studies have indicated that cKIT is expressed on cultured neonatal microglia (Santambrogio et al. 2001; Zhang and Fedoroff 1997) and that KIT ligand (stem cell factor, SCF) can promote their survival but inhibit CSF1-induced proliferation *in vitro* (Zhang and Fedoroff 1998). There is also some evidence that cKIT is induced upon microglial activation in response to a stab wound *in vivo* (Zhang and Fedoroff 1999). Conversely, FLT3 is not expressed on cultured neonatal microglia (Santambrogio et al. 2001) or isolated adult microglia (Anandasabapathy et al. 2011). Furthermore, mice deficient for FLT3 ligand have normal numbers of microglia and microglial numbers do not expand upon *in vivo* administration of FLT3 ligand. (Anandasabapathy et al. 2011). By qRT-PCR on mRNA isolated from whole left brain hemispheres we observed no difference in *cKit* expression between CSF1 over-expressing and control mice, and *cKit* expression did not change upon PLX3397 treatment (Supplementary Figure 6). We also examined *Flt3* expression and found a small but statistically significant decrease in CSF1 over-expressing mice compared to controls. However, *Flt3* levels did not change in CSF1 over-expressing or control mice following PLX3397 treatment (Supplementary Figure 6). If *cKit* and *Flt3* were normally expressed in adult microglia, we would have expected their expression to increase upon CSF1 over-expression and to decrease upon PLX3397 treatment similar to what was observed for the

known microglial markers *Iba1* and *Cd11b* (Figure 3A). Therefore, our qRT-PCR data suggest that *cKit* and *Flt3* are not normally expressed in adult microglia. Consequently, we hypothesize that the actions of PLX3397 at CSF1R are primarily responsible for the microglial apoptosis observed in PLX3397 treated mice. However, additional studies will be necessary to directly compare the actions of various CSF1R inhibitors in both the normal brain and in disease states.

Our studies indicate that PLX3397 does not impact *Gfap*⁺ astrocytes, *Rbfox3* (NeuN)⁺ neurons or *Mog*⁺ oligodendrocytes. Elmore et al. (2014) also found no differences in expression of neuronal or oligodendrocyte markers following PLX3397 treatment. Unlike our study, Elmore et al. (2014) did observe increases in *Gfap* mRNA following 7 days of PLX3397 treatment but their immunohistochemical studies with GFAP did not detect a difference in astrocyte numbers or morphology. As aging can impact astrocyte phenotypes (Sohrabji et al. 2013), it is possible that the observed differences in *Gfap* expression between these two studies following PLX3397 treatment are due to the utilization of 12 month-old mice by Elmore et al. (2014) while 4 week-old mice were analyzed in our study.

As CSF1 has been shown to modulate microglial activation phenotypes *in vitro*, we sought to determine if increased CSF1 levels impact basal and LPS-induced expression of several cytokines and polarization markers in microglia *in vivo*. In addition to microglia, other cell types in the CNS produce inflammatory mediators and respond to LPS (Carpentier et al. 2005; Verma et al. 2006). For this reason, we isolated CD11b⁺ cells for subsequent analysis to specifically examine microglial responses. Our data contrast with previous experiments that have primarily found that CSF1 acts synergistically with LPS to induce expression of several cytokines in monocytes/macrophages (Asakura et al. 1996; Evans et al. 1992; Hanamura et al. 1997; Sweet et al. 2002). One possible explanation is that the long-term exposure to CSF1 in our transgenics produces different phenotypes than does the relatively short exposures to recombinant CSF1 used in previous studies. Another explanation is that there could be differential impacts of CSF1 on microglia compared to other macrophage subsets. In support of this possibility, an *in vitro* and an *in vivo* experiment found that macrophage/monocyte subsets do exhibit differential responses to CSF1 and LPS, however neither of these studies specifically examined microglia (Chapoval et al. 1998; Kamdar et al. 1996). One *in vitro* study on microglial cultures observed that withdrawal of CSF1 promoted secretion of the pro-inflammatory cytokine IL-12 in response to LPS (Lodge and Sriram 1996), consistent with our studies indicating that CSF1 dampens microglial responses to LPS *in vivo*.

In summary, we have generated a genetic system for CSF1 over-expression in the CNS and used the system to test the hypothesis that increased CSF1 levels have pleiotropic influences on microglia. CSF1 over-expression was found to influence both microglial numbers and inflammatory responses. Furthermore, studies with PLX3397 indicate that the impact of CSF1 over-expression on microglial numbers can be counteracted by pharmacologic therapies, and that continued signaling through CSF1R is likely necessary for microglial survival. This genetic model should have utility for studying the impact of CSF1 over-expression in mouse models of human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

CSF1 over-expression in the *GFAP* compartment was found to modulate microglial proliferation and response to LPS. An inhibitor of CSF1R promoted microglial apoptosis, indicating that signaling through CSF1R is required for adult microglial survival.

Figure 1.

TRE-CSF1-IRES-GFP; GFAP-tTA mice over-express CSF1. A) TRE-CSF1-IRES-GFP mice were generated and crossed to GFAP-tTA mice to promote expression in the *GFAP* compartment. B) qRT-PCR demonstrates an approximately 4.3 fold increase in *Csf1* transcripts in brains from CSF1 over-expressing mice (CSF1, light shaded bar) compared to control TRE-CSF1 only mice (TRE, dark shaded bar), GFAP-tTA only mice (tTA, checkered bar), and mice lacking both transgenes (-/-; striped bar). *** $p < .001$, ns=non-significant ($p > .05$), Tukey's post-test following ANOVA. $n=3$ for TRE only and CSF1 mice

and n=4 for tTA and -/- mice. C) Immunofluorescence reveals the presence of detectable CSF1 protein (green) in a subset of GFAP⁺ (red) cells in CSF1 over-expressing mice (CSF1) but not in controls (CON). Arrow indicates an example of a GFAP⁺CSF1⁺ cell and arrowhead indicates a GFAP⁺ CSF1⁻ cell. Scale bars = 20 μ m D) Immunofluorescence for GFAP shows that in CSF1 over-expressing mice (CSF1), GFP fluorescence is detected in a subset of GFAP⁺ cells. Arrow indicates an example of a GFAP⁺GFP⁺ cell and arrowhead indicates a GFAP⁺ GFP⁻ cell. Scale bar= 10 μ m.

Figure 2.

CSF1 over-expression in the *GFAP* compartment promotes microglial proliferation and an increase in microglial numbers. A) Compared to controls (CON, dark shaded bars), CSF1 over-expressing mice (CSF1, light shaded bars) harbor significantly greater numbers of CD11b⁺ microglia even though they have a small but statistically significant decrease in brain weight. **** $p < .0001$, * $p < .05$, unpaired t-test. $n = 8$ CSF1 and $n = 10$ CON mice. B) Example Ki67/IBA1 double staining in the brainstem of control and CSF1 over-expressing mice. Arrow indicates an example of a Ki67⁺IBA1⁺ cell, arrowheads examples of

Ki67⁻IBA1⁺ cells and asterisk an example of a Ki67⁺ IBA1⁻ cell. Scale bar = 50 μ m. C)
The percentage of IBA1⁺ cells that were Ki67⁺ was quantified in the brainstem and midbrain. No proliferating microglia were observed in control mice but were readily observed in CSF1 over-expressing mice (CSF1, light shaded bars). **** $p < .0001$, unpaired t-test. n=4 control and n=4 CSF1 over-expressing mice.

Figure 3.

PLX3397 treatment reduces microglial numbers in both CSF1 over-expressing and control mice. A) qRT-PCR for the microglial markers *Iba1* and *Cd11b* on mRNA isolated from whole left hemispheres of brains from control (CON, dark shaded bars) and CSF1 over-expressing (CSF1, light shaded bars) mice that were treated with control chow (PLX-, solid bars) or PLX3397 containing chow (PLX+, checked bars) for one week. Values for each gene were normalized to control mice treated with control chow as one. Note discontinuous Y axis. n=5 control mice for each treatment and n=7 CSF1 over-expressing mice for each

treatment. B) Representative images of IBA1 IHC in control and CSF1 over-expressing mice treated with either control or PLX3397 containing chow for the indicated time. Brown indicates IBA1 immunostaining and nuclei are counterstained blue. Scale bar= 50 μ m. C) Quantification of the percent of cells that are IBA1⁺ (% microglia) in the indicated brain regions in control and CSF1 over-expressing mice treated with either control chow or PLX3397 chow for one week. n=9 for each group for the brain stem, n=8 for each group for the midbrain. Note that no IBA1⁺ cells were detected in the 40 $\times$ fields examined from the midbrain of PLX3397 treated control mice. D) Examples of TUNEL (green) and IBA1 (red) double staining in control mice treated without and with PLX3397 for 2 days. Arrowhead indicates an example of an apoptotic (TUNEL⁺ IBA1⁺) microglia. The merged image includes DAPI to visualize nuclei. Scale bar = 100 μ m. E) Quantification of the number of TUNEL⁺ IBA1⁺ cells in the brainstem of control and CSF1 over-expressing mice treated with either control chow or PLX3397 chow for 2 days. n= 3 per group. ANOVA followed by Bonferroni post-hoc tests were performed for the indicated comparisons. *** p<.001, * p<.05, ns=non-significant (p>.05).

Figure 4.

Microglia exposed to high levels of CSF1 are not basally polarized but are defective in inducing expression of certain genes in response to LPS. mRNA was isolated from microglia from control (CON, dark shaded bars) and CSF1 over-expressing (CSF1, light shaded bars) mice that were injected with PBS (-LPS) or with LPS (+LPS). qRT-PCR was performed to investigate relative expression levels of indicated M1 (top row) and M2 (bottom row) markers and cytokines. Values for each gene were normalized to control -LPS as one. $n=6$ for all groups with the exception of control +LPS where $n=7$. ANOVA followed by Bonferroni post-hoc tests for the indicated comparisons was performed. * $p<.05$, ** $p<.01$, *** $p<.001$ and ns=non-significant ($p>.05$). Note discontinuous Y axis for several genes.

Table 1

CSF1 transgenic mice genotyping primers

TRE-CSF1 genotyping 1	5'CACAACCATGGTGAGCAAGGG3'	5'CCTCGATGTTGTGGCGGATC3'
TRE-CSF1 genotyping 2	5'CCCAGGATGAGGACAGACAGGT3'	5'CACATTGCCAAAAGACGGCA3'
Internal control	5'GTGATCCCTCTACTTTTCTTCTGACTT3'	5'CGGAACGCAAATATCGCAC3'

Table 2

qRT-PCR primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
IL-1 β	TGTGCAAGTGCTGAAGCAGC	TGGAAGCAGCCCTTCATCTT
IL-6	ACTTCCATCCAGTTGCCTTC	GTCTCCTCTCCGACTTGTG
iNOS	TGACGCTCGGAAGTGTAGCAC	TGATGGCCGACCTGATGTT
TNF α	TGTAGCCACGTCGTAGCAA	AGGTACAACCCATCGGCTGG
ARG1	AGCCAATGAAGAGCTGGCTGGT	AACTGCCAGACTGTGGTCTCCA
IL-10	CCTGGGTGAGAAGCTGAAGA	TTTTCACAGGGGAGAAATCG
TGF β	TGACGTCAGTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
YM1	AAGCTCTCCAGAAGCAATCCT	TCAGAAGAATTGCCAGACCTGT
18s	CGGGTGCTCTTAGCTGAGTGTCCCG	CTCGGGCCTGCTTTGAACAC
CSF1	TTGCCAAGGAGGTGTGAGAACACT	AAGGCAATCTGGCATGAAGTCTCC
IBA1	TGATGAGGATCTGCCGTCCAACT	TCTCCAGCATTGCTTCAAGGACA
CD11b	AAAGGCTGTAAACCAGACAGGTGC	ACAGGCCCAAGGACATATTCACAG
FLT3	CCAGTCAGCGTTGGTGA	GGGTCAATTCATACTCTTCTTGC
cKIT	CTTATTGAGAAGCAGATCTCGGA	GGGTCTCTGGGTGGG
GFAP	CATGCAAGAGACAGAGAGTGGT	AGTCGTTAGCTTCGTGCTTGGCTT
RBFOX3 (NeuN)	CCTGTGGTAGGAACAGTCTATG	GGTGGTGCAGCTCGAAA
MOG	GCTTCTTCAGAGACCACTCTTAC	GTAGGCACAAGTGCGATGA
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG